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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 :
C12Q 1/56, G01N 33/86

A1

(11) International Publication Number: WO 91/01382

(43) International Publication Date: 7 February 1991 (07.02.91)

(21) International Application Number: PCT/SE90/00478

(22) International Filing Date: 4 July 1990 (04.07.90)

(30) Priority data:
8902532-4 14 July 1989 (14.07.89) SE

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(81) Designated States: AT (European patent), AU, BE (European patent), CA, CH (European patent), DE (European patent)*, DK (European patent), ES (European patent), FR (European patent), GB (European patent), IT (European patent), JP, LU (European patent), NL (European patent), NO, SE (European patent), US.

Published
With international search report.

(54) Title: A METHOD FOR DETERMINING THE FUNCTIONAL ACTIVITY OF FREE PROTEIN S OR PROTEIN C IN A PLASMA SAMPLE

(57) Abstract

There is described a method for assaying the functional activity of free Protein S or Protein C in a plasma sample, by adding to the plasma sample a coagulation enzyme, FIX_a, which enzyme promotes the formation of coagulation enzyme FX_a optionally together with further coagulation factors, whereafter the sample is incubated and the quantity of thrombin formed from prothrombin, this process being promoted by FX_a, is measured in a known manner. The level of functionally active Protein S or Protein C, which is inversely correlated to the amount of thrombin formed, is then determined on the basis f the quantity of thrombin measured, in a known manner with the aid of a standard.

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A method for determining the functional activity of free Protein S or Protein C in a plasma sample.

The present invention relates to the assay of components in the blood coagulation and fibrinolysis systems, and particularly to the assaying or determination of the functional activity of certain proteins included in these systems, namely the plasma proteins designated Protein C and Protein S.

One purpose of the blood coagulation or clotting process is to effectively stop bleeding. This process involves a complicated so-called enzymatic cascade of enzyme-activating reactions initiated by contact activation, e.g. through an injured blood vessel, of a proenzyme, Factor XII (the word Factor is abbreviated hereinafter to F, as is normal practice) to an active enzyme $FXII_a$ (the suffix "a" stands for active and this labelling method is used generally in the following text). $FXII_a$ catalyzes a subsequent activation reaction of proenzyme to enzyme and the blood coagulum or blood clot is finally formed through a series (cascade) of enzyme activations, by the conversion of soluble fibrinogen to insoluble fibbrin. The many activation stages, i.e. the cascade of reactions, contribute to the rapid formation of the blood clot, so as to stop bleeding rapidly.

The reverse process, i.e. the lysis of formed blood clots, so-called fibrinolysis also comprises a similar enzyme reaction cascade, wherein plasmin is formed in the ultimate stage of the cascade. The plasmin formed functions to degrade the clot, i.e. the fibrin, quickly

into smaller, soluble fragments, by proteolysis.

These systems incorporate a large number of factors, as will be described in more detail herebelow.

Enhanced or reduced quantities of one or more of these factors in the blood, due to acquired or inherited disorders in the coagulation and/or fibrinolysis systems will often lead to pathalogical conditions, which may be fatal, and in the case of the individual may, for instance, mean a predisposition to the formation of arterial and/or venous thrombosis (or blood clots). Reduced quantities of functionally active antithrombin and plasminogen and enhanced quantities of FVII, fibrinogen and plasminogen activator inhibitors PAI-1 are examples of disorders which can lead to thrombosis (or blood clots).

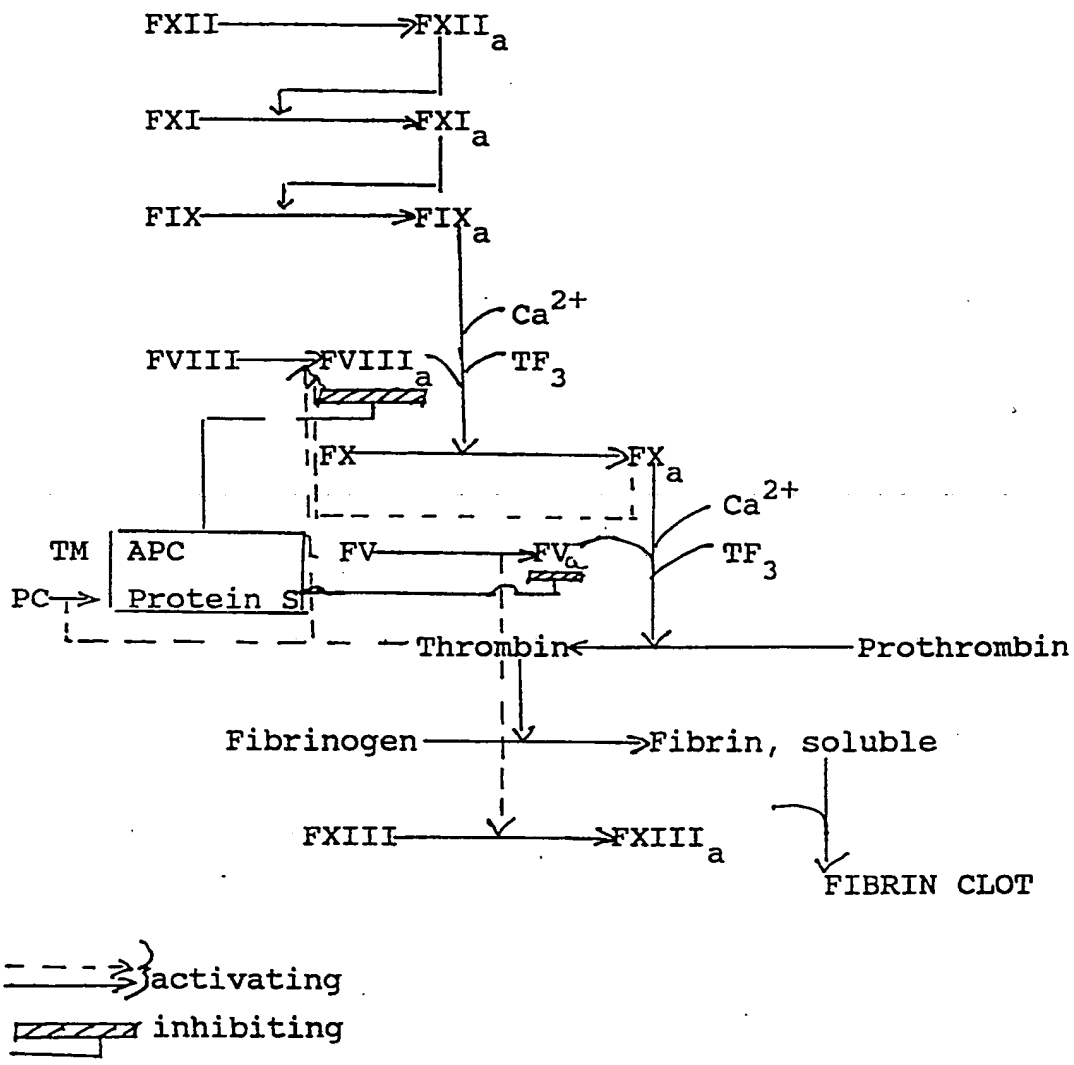
Recently, two novel proteins, designated Protein C (PC) and Protein S (PS) have been identified in plasma. Similar to the well-known antithrombin, these proteins, which are vitamin K dependent, have an anticoagulating activity, said proteins coacting such that Protein S (molecular weight 80 kD) will stimulate the activity of activated Protein C and therewith both counteract clot formation and promote degradation of clots that have already formed. Reduced quantities of these proteins, or of one of said proteins, will also lead to disorders in the coagulation and fibrinolysis systems. The significance of Protein S has been confirmed by studies carried out on the protein. On the basis of these studies, it is believed that in the case of patients afflicted with deep venous thrombosis and younger than 50 years of age, the clinically manifested thrombosis in 5-8% of cases is due to an inherited deficiency of

Protein S, whereas an inherited deficiency of antithrombin is believed to be responsible for only about 3% of all cases.

Considerable benefit would therefore be gained if it were possible to assay, or determine the content of, both or one of the proteins as a matter of routine, since this will enable the predisposition of an individual to the formation of such thrombosis to be established more easily and to enable prophylaxis and/or therapeutical treatment to be administered in good time.

These plasma proteins, PC and PS, influence the formation of FX_a and thrombin, by coacting to cleave the cofactors FV_a and $FVIII_a$, necessary for effecting formation of thrombin. In order to facilitate an understanding of the function of Protein S and Protein C (in an activated form), a brief description is given below of the essential parts of the coagulation system, of which the stages from and including activation of FXII are shown in Schedule 1:

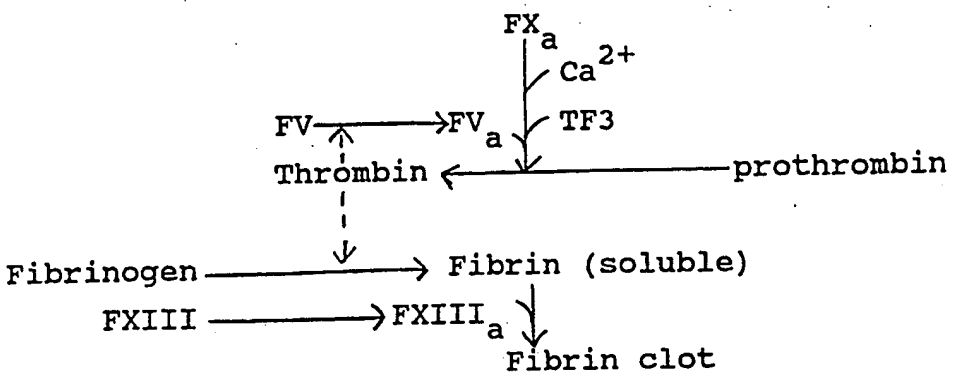
Schedule 1



As will be seen from this schedule (1), the inactive protein prothrombin (proenzyme) is converted to active thrombin (enzyme), through the influence of activated FX, i.e. FX_a, Ca^{2+}, a phospholipid designated TF3 (thrombocyte factor 3) which is exposed on the surface of activated thrombocytes, and a helper-protein FV in its activated form FV_a. The thrombin cleaves the fibrinogen enzymatically, such as to obtain fibrin, which forms the structural framework of the clot, by being cross-linked with the aid of the enzyme $FXIII_a$. These processes are illustrated in Schedule 2 below, which

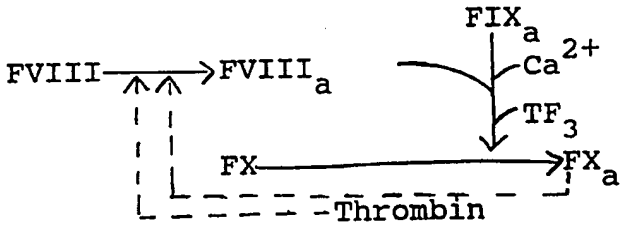
thus forms part of Schedule 1.

Schedule 2



In turn, FX_a has been formed by inactive FX through the action of FIX_a and with the aid of TF_3, Ca^{2+} and a further helper protein FVIII in the active form $FVIII_a$. These processes are illustrated in Schedule 3 below, which also forms part of Schedule 1.

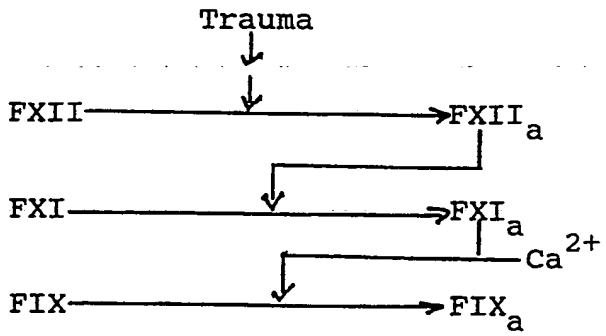
Schedule 3



As will be seen from these schedules, thrombin is self-regulating with respect to the quantity formed, by contributing to the activation of FV_a (Schedule 2) and, together with FX_a, also to the activation of $FVIII_a$ (Schedule 3), these active factors being necessary for effective thrombin formation, and also by contributing to the activation of Protein C which, in active form, together with Protein S decreases thrombin formation by cleaving the factors $FVIII_a$ and FV_a, as will be explained here below.

The enzyme FIX_a is formed by inactive FIX through the action of FXI_a and Ca^{2+}. FXI_a has been obtained by FXI through the action of $FXII_a$. $FXII_a$ is formed by FXII as a result of vessel injury or the like, which consequently is the initiating factor in the clotting system. This is illustrated in Schedule 4 below, which also forms part of Schedule 1.

Schedule 4

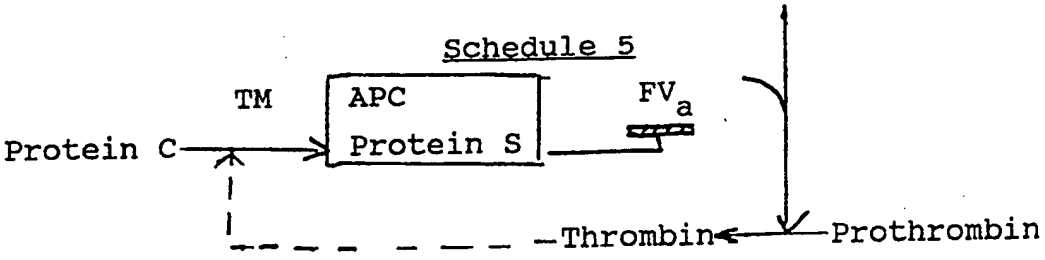


In addition to the processes disclosed in Schedule 2 and Schedules 3 + 4, the formation of thrombin is promoted by activation of FIX and FX to FIX_a and FX_a respectively, via the extrinsic system with the assistance of a tissue factor, tissue thromboplastin, which supplies helper-protein and phospholipid, and by the action of the enzyme FII_a in the presence of Ca^{2+}.

The activated forms of the coagulation and fibrinolysis factors do not normally occur in the blood, but are only formed when needed. The blood contains a number of proteins, so-called enzyme inhibitors, which guard against erroneous activation. One well known inhibitor is antithrombin, which binds thrombin to a complex lacking proteolytic activity.

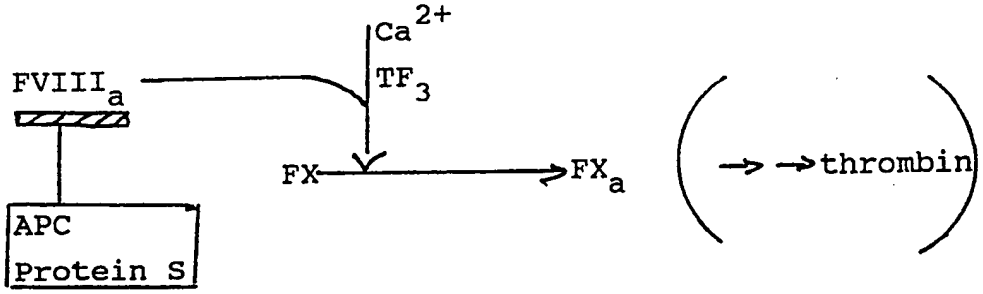
Protein C (PC) and Protein S (PS), to which the present invention relates, also act as inhibitors or anticoagulants, the effect of these proteins being based on the activation of Protein C to active Protein C, APC, which cleaves FV_a with the assistance of Protein S. This reduces the conversion of prothrombin to thrombin (Schedule 5) dramatically, and thus the formation of clots.

Schedule 5



In addition, APC exerts, together with PS, a cleaving effect on $FVIII_a$, which also assists in the anticoagulating effect, by reduced formation of FX_a. This process is illustrated in Schedule 6 below.

Schedule 6



As will be seen from these schedules, the activation of Protein C is controlled by some kind of feedback mechanism which incorporates thrombin for the purpose of restricting clot formation. When clots are formed in a blood vessel, thrombin is passed from the clot to the vessel wall, where thrombin is bound to thrombomodulin (TM), which is a protein on the endothelial cell sur-

no longer have a coagulating effect, i.e. it is no longer able to cleave the fibrinogen, and instead the complex TM/thrombin quickly activates Protein C to APC which, in the form of a complex with Protein S, inhibits coagulation by cleaving FV_a and $FVIII_a$. This latter complex thus has an important anti-clotting effect.

As mentioned earlier, APC also promotes clot lysis. This takes place indirectly, by protection of the tissue plasminogen activator (t-PA) against the effect of inhibitors. In this way, a high conversion rate of plasminogen to plasmin is maintained, through the effect of t-PA. Plasmin dissolves the fibrin clot, by cleaving the clot into smaller, soluble fragments.

Too low contents of Protein C and/or Protein S, will render an individual predisposed to thrombotic diseases, as a result of insufficient moderation of thrombin formation, which results in excessive formation of fibrin with the accompanying risk of thrombosis. Consequently, there is a need of a simple and safe process by means of which the levels of Protein C and/or Protein S in a sample can be measured in a routine fashion, so as to enable prophylax and/or therapeutic treatment of thrombosis to be improved. It is the functional activity of Protein C and/or Protein S which shall be determined in particular, since about 10% of all individuals suffering from a Protein C deficiency exhibit normal immunological levels of Protein C, despite the fact that the functional activity is greatly reduced or totally lacking. With regard to Protein S, the concentration of which in plasma is about 22 µg/ml, the greater part thereof, about 60%, is bound to a protein within the complementary system, the C4b-binding protein, and is functionally inactive. The remaind-

er, about 10 μg/ml, which is present in a free state in plasma, is responsible for the biological activity, i.e. is bound to activated Protein C to form an active complex. Since individuals suffering from a Protein S deficiency can exhibit normal levels of bound Protein S, despite lacking or exhibiting very low levels of free Protein S, it is the functional activity which shall be measured also in the case of Protein S deficiency.

Hitherto, however, no satisfactory process has been developed for assaying the functional activity of primarily Protein S.

The functional activity of Protein S has, admittedly, been the subject of earlier assaying methods, although not as matter of routine, and the known methods are not sufficiently reliable with respect to distinguishing between various levels of biological activity. Those methods known at present are based on recording the time at which a fibrin clot occurs. These methods are designated APTT-based or FX_a-based coagulation methods, the abbreviation "APTT" standing for "Activated Partial Thromboplastin Time". In this regard, plasma is activated in a first stage with a reagent containing phospholipid and a contact activator, such as kaolin or ellaginic acid, thereby initiating the calcium-dependent coagulation reactions (see Schedule 4), i.e. $FXII_a$ and FXI_a. Ca^{++}-ions are added in a second stage, wherewith FIX, FX and prothrombin are activated to varying degrees. A known quantity of one or more coagulation factors is also added, for the purpose of determining the Protein S activity.

The coagulation sequence initiated in stage 1 thus

proceeds to completion, so as to form a blood clot. The time (in seconds) taken for the clot to form (APTT) is recorded and correlated to the functional activity of Protein S, with the aid of standard samples. Generally, an increasing content of free Protein S in plasma will require a longer time for a blood clot to form, since larger quantities of the coagulation inhibiting APC/Protein S-complex are formed.

The activating coagulation factors added to the system in these known methods are activated Protein C (APC) and FX_a [see P. COMP and C. ESMON, "Recurrent Venous Thromboembolism in Patients with a Partial Deficiency of Protein S", New Eng. J. Med. 311, 1525-1528 (1984), and B. DAHLBÄCK, "Inhibition of Protein C_a Cofactor Function of Human and Bovine Protein S by C4b-Binding Protein", (1986)]. According to A. d'ANGELO, S. VIGANO-d'ANGELO, C. ESMON, P. COMP, "Acquired Deficiencies of Protein S. Protein S Activity During Oral Anti-coagulation, in Liver Disease and in Disseminated Intravascular Coagulation". J. Clin Invest 81, 1445-1454 (1988) first free Protein S in plasma is extracted with the aid of a specific monoclonal antibody, whereafter APC and FX_a are added to the extracted protein. In K. SUZUKI and J. NISHIOKA, "Plasma Protein S Activity Measured Using Protac, a Snake Venom Derived Activator of Protein C", Thromb Res. 49, 241-251 (1988), there is used instead of APC a substance which activates PC in plasma to APC, namely Protac® C (from Pentapharm, Switzerland), the active component of which is a selective snake venom enzyme from Agkistrodon Contortrix contortrix.

These known methods, however, have several drawbacks. For instance, the low resolution, i.e. the discrimin-

ation of different levels of free Protein S, is limited. Typically, the coagulation time is extended by only 10 s within the range of 70-200% free Protein S, the coagulation time for normal plasma, i.e. 100% free Protein S, lying within a range of 40-80 s, depending on the method used. Furthermore, these methods are difficult to carry out on a routine basis, e.g. in coagulation laboratories, and require thorough standardization and are deficient with respect to accuracy.

Accordingly, it is an object of the present invention to provide a method for assaying the levels of functionally active Protein S or Protein C in blood plasma, which can be carried out simply and which will essentially circumvent the drawbacks associated with known methods.

In accordance with the invention, this object is achieved with a method comprising the addition of the coagulation enzyme FIX_a, this enzyme promoting the formation of the coagulation enzyme FX_a, optionally together with further coagulation reagent(s), to a plasma sample whose content of functionally active Protein S or Protein C shall be determined, incubating the sample and measuring, in a known manner, the amount of thrombin derived from prothrombin, this process being catalyzed by FX_a, and measuring the content of functionally active Protein S or Protein C, which is inversely correlated to the amount of thrombin formed, on the basis of the measured quantity of thrombin in a known manner with the aid of a standard.

Thus, the quantity of thrombin formed constitutes a measure of the quantity of functionally active Protein S or Protein C present. Methods for assaying the throm-

bin content in plasma are well known, and the invention is not limited to any particular measuring method. All methods known at present and also those methods which may be devised in the future are usable. Examples of suitable measuring methods include substrate cleaving based, preferably photometric, measuring processes and coagulation-based measuring processes.

In accordance with the invention, it is found that the photometric substrate-cleaving method is well-suited for determining the functional activity of Protein S and Protein C on the basis of the quantity of thrombin formed, and this method provides considerably greater resolution than that achieved with the known coagulation-based methods, particularly with respect to Protein S. According to one preferred embodiment of the invention, photometric measurement of the quantity of thrombin formed is therefore applied, wherein a selective thrombin substrate, preferably a chromogenic thrombin substrate, is used. Such substrates are normally based on amino acids or, preferably, on short peptides which are provided with a group (marker) which can be readily and selectively cleaved by thrombin and can be readily measured, e.g. photometrically, when a chromogenic marker, such as p-NA (p-nitroaniline) is used. These substrates are available commercially, for instance, from Kabi Diagnostica, Mölndal, Sweden and/or can be easily prepared by the person skilled in this art.

It is also surprisingly found, in accordance with the invention, that by suitable selection of the selective thrombin substrate, the inventive method can be carried out optionally in either two stages or in one single stage. It is thus surprising and beneficial that the

method can be carried out in one single stage when a suitable selective substrate is chosen. The substrates which make a single-stage process possible are, for instance, (S-2846) (from KabiVitrum AB, Sweden) and H-D-CHG-Ala-Arg-pNA (Nycomed Th-1) (from Nycomed AS, Norway). The known thrombin substrate H-D-Phe-Pip-Arg-pNA (S-2238) (from KabiVitrum AB, Sweden), on the other hand, is not suited for a single-step process, due to insufficient resolution among other things.

The inventive method also enables the quantity of thrombin formed to be established with the aid of coagulation methods in a known manner. The coagulation sequence is therewith permitted to proceed to completion and the time taken for a clot to form on the plasma sample is measured and compared with corresponding times for plasma with known functional activity of Protein S or Protein C. The process can be carried out in either one stage or in two stages.

The present method is based on an addition of FIX_a for promoting activation of FX to FX_a, instead of adding FX_a directly, as is often the case when practicing prior art methods. The FIX_a used in accordance with the invention is normally of mammal origin, e.g. from bovine. FIX_a from humans or pigs can also be used with advantage.

Addition of FIX_a in accordance with the invention provides the advantage of enabling activation of FX to FX_a to be controlled and, indirectly, also of enabling the inactivating effect of APC/PS on formed FV_a and $FVIII_a$ to be optimized, which in turn greatly influences the formation of thrombin. The strong influence of FIX_a on the process, for instance in comparison with the addi-

tion of FX_a at corresponding plasma contents, could not be foreseen. Neither has the addition of FIX_a for the purpose intended with the invention been earlier described.

The inventive method can be further improved by adding certain other coagulation factors, in addition to FIX_a. Thus, according to one preferred embodiment, the method is carried out while adding activated Protein C, i.e. APC. The APC used is normally of mammal origin, such as from bovine (bovine APC) or pigs (porcine APC) or preferably from humans (human APC). Usable APC can be prepared from plasma or by recombinant techniques, and is normally highly purified. The earlier mentioned commercial preparation Protac® C including a snake venom enzyme can be used instead of APC. This enzyme activates Protein C to APC and thus promotes indirectly formation of the APC/PS complex, the effect of which has been discussed earlier.

According to a further suitable embodiment of the invention, FV_a is also added to the plasma sample. The addition of FV_a is used to control the rate at which thrombin is formed, and the conversion of prothrombin to thrombin can be effected more quickly and more efficiently by the suitable addition of FV_a. There is preferably used an FV_a of mammal origin, such as human origin, bovine origin or porcine origin, and preferably bovine origin, FV_a prepared from plasma or via recombinant techniques.

Phospholipid is another coagulation reagent which can be appropriately added to the plasma sample when carrying out the present invention. This addition is able to accelerate the activation of FX to FX_a and prothrombin

to thrombin. The choice of the phospholipid source is not critical. For instance, both commercial APTT-reagents, with or without contact activator, such as kaolin or ellaginic acid, and synthetic phospholipids or phospholipid mixtures have been found usable.

In certain cases, the addition of prothrombin, FX and/or FVIII has been found advantageous. With respect to all coagulation proteins used in accordance with the present method, it will be understood that these coagulation proteins are not limited with respect to species origin and that they are embraced by the invention irrespective of whether they are extracted from plasma or prepared via recombinant techniques from native or molecular biologically modified variants thereof. With regard to species origin, however, mammal origin is normally preferred, and then particularly human, bovine or porcine origin.

The proportions in which the majority of components are present when applying the present method are not particularly critical. However, in order to facilitate an understanding of the invention, suitable concentration ranges for a number of components have been set forth in the following Table 1.

Table 1

Component/ conditions	Final Content in Test
Plasma	0.02-10 percent by volume, particularly 0.1-2 percent by volume* up to 50 percent by volume**
FIX_a	$1 \cdot 10^{-15}$-$1 \cdot 10^{-6}$ mol/l***
APC	$5 \cdot 10^{-11}$-$1 \cdot 10^{-7}$ mol/l, preferably $1 \cdot 10^{-10}$-$1 \cdot 10^{-8}$ mol/l
FV_a	$1 \cdot 10^{-12}$-$1 \cdot 10^{-9}$ mol/l, preferably $2 \cdot 10^{-10}$-$1 \cdot 10^{-11}$ mol/l
Phospholipids	$1 \cdot 10^{-6}$ - $3 \cdot 10^{-4}$ mol/l, preferably $3 \cdot 10^{-6}$-$1 \cdot 10^{-4}$ mol/l
Ca^{2+}	10^{-4}-$5 \cdot 10^{-2}$ mol/l, preferably 10^{-3}-10^{-2} mol/l****
Thrombin substrate, e.g. chromogenic	$2 \cdot 10^{-6}$-$2 \cdot 10^{-3}$ mol/l, preferably 10^{-4}-10^{-3} mmol/l
pH	6.5-9.5, preferably 7-8.5
Ion strength (I)	0-0.6, preferably 0-0.25
Protac® C	$5 \cdot 10^{-3}$-1.5 U/ml
Prothrombin	$1 \cdot 10^{-9}$-$2 \cdot 10^{-6}$ mol/l
FX	$1 \cdot 10^{-13}$-$5 \cdot 10^{-8}$ mol/l
FVIII	$1 \cdot 10^{-4}$-$5 \cdot 10^{-1}$ IU/ml

*Thrombin substrate method, e.g. chromogen method.

**Coagulation method.

***Higher contents in test without an addition of FVIII and/or FV_a.

****Calcium may be added in the form of $CaCl_2$ or some

other suitable salt.

In order to maintain the pH in the range preferred, the component-solutions are prepared in buffer solutions, using conventional buffer types, such as Tris-buffer.

The inventive method involves incubating the sample at a temperature of 18-45°C, preferably 35-40°C and more preferably at 37°C, for a short period of time, e.g. 0.5-15 minutes, suitably 1-10 minutes and particularly 0.5-6 minutes, whereafter the functional activity of Protein S or C is measured, all in accordance with a so-called single-stage method. In the case of systems having pronounced short reaction times, such as preferably coagulation-based methods, it is preferred to heat the sample in an introductory stage, optionally together with a coagulation factor, to the same temperatures as those recited above, over a short period of time, normally 2-3 minutes, before adding FIX_a and optionally also other coagulation factors to the sample. In certain cases, it may be advantageous to apply a two-stage method incorporating two incubation periods, for instance when Protac® C is used to activate Protein C in the plasma sample.

It may also be beneficial to use in the present method polyclonal or monoclonal antibodies against a coagulation factor, with the intention of eliminating the influence of this factor. For instance, the biological activity of FVIII can be blocked by adding anti-FVIII:C antibodies.

The aforesaid applies generally for assaying the functional activity of Protein S. When the present method is applied for assaying the functional activity of

Protein C, it may be necessary to make some modification. For instance, Protein S, preferably of human origin, is added to the plasma sample either before or after activation of Protein C in the plasma sample, this activation advantageously being effected with Protac® C, as disclosed above. FIX_a and optionally other coagulation factors is, or are, then added in a second stage.

The invention will be explained in more detail with the aid of the following examples, which are intended solely to illustrate the invention without limiting the scope thereof. These examples include references to the accompanying drawings, of which Figure 1 illustrates the assaying of Protein S with a chromogenic single-stage method in the presence of FIX_a or FX_a; Figure 2 illustrates the effect of anti-FVIII:C antibodies and bovine FX when assaying Protein S; Figure 3 illustrates the effect of various substrates in a single-stage method for assaying Protein S; Figure 4 illustrates a single-stage coagulation-based method for assaying Protein S on the basis of a FIX_a addition; and Figure 5 illustrates a FIX_a-based chromogenic method for assaying Protein C.

Example 1

(a) A plasma sample was diluted 1:15 in Tris-buffer I. 100 µl thereof were mixed with 100 µl of human APC dissolved in Tris-buffer I, 200 µl of a chromogenic substrate H-D-Ala-Pro-Arg-pNA (S2846) from KabiVitrum Diagnostica, Sweden) and 200 µl in total of human FIX_a dissolved in Tris-buffer II, $CaCl_2$, bovine FV_a dissolved in Tris-buffer I and a phospholipid mixture comprising 40% cholesterol, 40% phosphatidylcholine and

20% phosphatidyl serine.

The Tris-buffer I comprised 0.05 mol/l Tris-HCl having a pH of 7.4 and an ion strength (I) of 0.15, and 0.2% bovine serum albumin (BSA).

The Tris-buffer II comprised 0.05 mol/l Tris-HCl, pH 8.0, I = 0.15 and 0.2% BSA. The concentrations of the starting solutions were such as to obtain the final concentrations given for the reactants in Table 1.

Table 1

Component	Added Volume	Final Content
Plasma	100 µl	1 percent by volume
Human APC	100 µl	1 nmol/l
H-D-Ala-Pro-Arg-pNA (S-2846)	200 µl	0.33 mmol/l
FIX_a	200 µl	0.1 nmol/l
FV_a		75 pmol/l
$CaCl_2$		5 mmol/l
Phosphlipid		12 µmol/l
	600 µl (total volume)	

The mixture was incubated for 4 minutes at 37°C, whereafter 20%-acetic acid was added, with the intention of interrupting the reaction processes. The absorbance of the sample was then determined at 405 nm (A_{405}) with standard equipment, such as a photometer, e.g. Hitachi 100-20. A_{405} was also determined for standard samples prepared by mixing normal plasma, the Protein S content of which was set at 100%, and Protein S-deficient plasma, the Protein S content of which was set at 0%. A_{405} was also measured for such plasma as

that containing 100% PS and 0% PS respectively.

The values obtained for A_{405} are plotted against %PS in Figure 1 (- ■ -). It will be seen from these values that very good resolution was achieved, which is reflected in the wide difference between absorbance at 100% and at 0%, this difference being designated ΔA_{405} (0-100%), namely roughly 0.85. It will also be seen from Figure 1 that the Protein S content is inversely correlated to the quantity of thrombin formed, i.e. that increasing quantities of PS result in a considerable reduction in the formation of thrombin.

(b) In Example 1 (a), the assay was carried out with the addition of APC to the plasma sample. By way of comparison, an analogous assay was carried out without adding APC, wherein a substantially smaller, not significant difference ΔA_{405} (0-100%) was obtained, i.e. the test had a low resolution degree. This is evident from the plotted values (- ■ -) in Figure 1. The measured absorbance remained substantially unchanged at a high level when carrying out the assay according to Example 1 (a) subsequent to initial incubation of the sample with antibodies against Protein S (designated PS-Ab), whereby the biological activity of PS, and therewith also of the APC/PS-complex, was eliminated. The values obtained are also plotted (-x-) in Figure 1.

(c) By way of comparison with known techniques, an assay was also carried out analogously with 1 (a) above, with the addition of human APC and without the addition of human APC, but with the substitution of FIX_a with bovine FX_a (final concentration 0.4 pmol/l and 5-min. incubation). The A_{405}-values obtained are also plotted in Figure 1 (- ▲ - and - Δ - respective-

ly). It will be evident herefrom that no significant values of A_{405} (100-0%) were obtained and that the thrombin formation was constantly relatively low.

Although the incubation time was shortened somewhat when the FX_a-addition was increased to a final proportion of 40 pmol/l, the resolution was impaired still further. A slightly better result was achieved when the amount of APC addition was increased, although not even a three-fold increase in the APC-addition gave a ΔA_{405} (0-100%)-value higher than 30% of the value obtained in accordance with Example 1 (a).

Example 1 illustrates a one-stage chromogenic method for assaying the functional activity of PS in plasma. The surprisingly high effect achieved when adding FIX_a to the sample is clearly evident from the results obtained (set forth in Figure 1). It is admittedly known that APC will degrade the cofactors FV and FVIII via proteolytic cleaving (W. KISIEL, W. CANFIELD, L. ERICSSON, E. DAVIE, Anticoagulant Properties of Bovine Plasma Protein C Following Activation by Thrombin. Biochemistry 16, 5824-5831 (1977); and R. MARLAR, A. KLEISS, J. GRIFFIN, "Mechanism of Action of Human Activated Protein C, a Thrombin-Dependent Anticoagulant Enzyme". Blood 59, 1067-1072 (1982)), which process, however, is not effective before FV and FVIII have been activated respectively to FV_a and $FVIII_a$ by thrombin and/or FX_a. Consequently, it is quite surprising that the addition of a preformed FX_a will result in considerably lower APC/PS activity than the addition of FIX_a (according to the invention), which assists in the formation of FX_a during incubation, since activation of FVIII via the influence of FX_a/thrombin can take place in both instances.

The following Example illustrates the significance of the cofactor $FVIII_a$ to thrombin formation by blocking the activity of the cofactor.

Example 2

One volume of normal plasma (100% PS) was preincubated at 37°C for 15 minutes with one volume of 0.2 µg/ml of monoclonal anti-FVIII:C antibodies 2A3 (from Kabi AB, Sweden). These antibodies bind specifically to FVIII, which is thereby inactivated. Analogously with Example 1 (a), but with a plasma content of 1.7% and with an FIX_a-addition, A_{405} was determined for plasma samples having 0, 50 and 100% PS respectively after incubation for 4 minutes at 37°C, and with an addition (final content 0.02 U/ml) of bovine FX or without such an addition. The values obtained are plotted in Figure 2. It will be evident herefrom that in the absence of $FVIII_a$, APC/PS will still produce an effect when adding FX (- ■ -), namely through the influence of APC/PS on the FV_a supplied.

In comparison with Example 1 (c), comprising direct addition of FX_a (Figure 1; - ▲ -), it would again appear advantageous to form FX_a during the incubation period, from FX that has been added instead of adding FX_a directly to the system. (Figure 2; - ■ -).

Example 3

Analogously with Example 1 (a), A_{405} was determined for normal plasma (100% PS) and Protein S deficient plasma (0% PS). Instead of using APC, however, Protac® C (final content 0.17 U/ml) was used, which activates

(final content 0.17 U/ml) was used, which activates Protein C to APC upon incubation for 2 minutes at 37°C, and while using 12 pmol/l of FIX_a and applying an incubation time of 4.5 minutes. The results obtained are set forth in Table 2.

Table 2

	A_{405} + Protac® C	A_{405} - Protac® C
Protein S-deficient plasma (0% PS)	0.87	-
Normal plasma (100% PS)	0.18	1.6

It will seen from these values that Protein S exhibits high activity in the presence of APC. It is not necessary to supply APC initially, since APC can be formed in vitro through the action of Protac® C.

Example 4

A_{405} for normal plasma and Protein S-deficient plasma was assayed analogously with Example 1 (a), although with a final proportion of 18 µmol/l of the synthetic phospholipid mixture. Analogous assays were also made with the aid of an APTT-reagent, Cephotest® (from Nycomed AS, Norway), which also includes a contact activator, namely ellaginic acid, instead of the phospholipid mixture, which in this case comprised an extract from bovine brain. The values obtained are set forth in Table 3.

Table 3

COMPARISON BETWEEN CEPHOTEST® AND SYNTHETIC PHOSPHOLIPID (PL) MIXTURE

	A_{405}	
% Protein S	Cephotest®	PL-mixture
0	0.48	0.44
100	0.15	0.18

These A_{405} values exhibit good agreement, which indicates that different phospholipid sources can be used, including mutually different compositions of synthetic phospholipids and also mutually different contact activators.

Example 5

Analogously with Example 1 (a), plasma containing different percentages of PS were incubated in a single-stage method with the use of three different substrates and additions of bovine FIX_a.

The final sample had a plasma content of 1.1 percent by volume, a substrate content of 0.3 mmol/l and contained 0.1 nmol/l FIX_a, 75 pmol/l FV_a and 2 nmol/l APC. The substrates tested consisted of S-2846 and S-2238 (both from KabiVitrum Diagnostica, Sweden) and Nycomed Th-1, the incubation times being 4 min., 5 min. and 4 min. respectively.

The results are set forth in Figure 3. It will be seen from these results that S-2846 (- □ -) is a far better substrate than S-2238 (- ◆ -). It will also be seen from Figure 3 that a good effect was also obtained with the substrate Nycomed Th-1, (- O -) (from Nycomed AS, Oslo, Norway).

For comparison purposes, the aforesaid method was also carried out as a two-stage method with the use of substrates S-2846 and S-2238, i.e. the plasma sample to which APC was added was incubated prior to the substrate addition. In the two-stage method, the ΔA_{405} (0-100%)-values obtained were 0.8 and 0.6 respectively, i.e. no pronounced difference in effectiveness was observed. The following substrates were also tested in a single-stage method and found to be useful, (ΔA_{405} (0-100%) = 0.2-0.5).

Chromozym Th:	Tosyl-Gly-Pro-Arg-pNA (from Pentapharm AB, Basel, Switzerland).
Spectrozym Th:	H-D-CHT-Ala-Arg-pNA (from American Diagnostica, Greenwich, U.S.A.).
CBS 34.47:	H-D-CHG-But-Arg-pNA (from Diagnostica Stago, Asnieres, France).
Thrombin substrate from Behring:	H-D-CHA-But-Arg-pNA (from Behringwerke AG, Marburg, Federal Republic of Germany).

wherein

CHT = cyclohexyltyrosine
CHG = cyclohexylglycine
But = α-amino butyric acid
CHA = cyclohexylalanine
pNA = p-nitroanilide

Tosyl = p-toluene sulphonyl

Example 6

100 µl of human APC were added to 100 µl of a plasma sample diluted to 1:1 in a 0.9%-NaCl solution and the sample was incubated for 2 minutes at 37°C. 100 µl totally of bovine FIX_a, bovine FV_a, Cephotest® and $CaCl_2$ were then added. The contents of the starting solutions used were such as to obtain the following final concentrations:

Table 4

Component	Final Content
APC	15 nmol/l
FIX_a	0.4 nmol/l
FV_a	75 pmol/l
Cephotest®	4.2 percent by volume
Ca^{2+}	4.2 mmol/l

Plasma samples with 0, 50 and 100% PS, i.e. Protein S-deficient plasma (0%), normal plasma (100%) and a 1:1 mixture (50%) thereof were used in the assay, which was carried out as a single-stage coagulation-based method. The coagulation times of these samples were registered and plotted (Figure 4; - ■ -) against % PS for the sample.

Analogously with the aforegoing, the coagulation time was also recorded for plasma samples which had not been activated with APC (see Figure 4; - Δ -).

As will be seen from Figure 4, an almost 60 second

extension of the coagulation time was achieved for 100% PS in comparison with 0% PS, this effect being a considerable improvement on the effect achieved with earlier known methods which lie closest to the afore-described method with respect to plasma contents (P. Comp et al, loc cit; B. Dahlbäck, loc cit). No difference (i.e. no extension) in coagulation time between 0 and 100% PS in the above method is obtained in the absence of an APC-addition (- Δ -).

Example 7

A plasma sample to which human Protein S was added was diluted 1:15 with Tris-buffer I. 100 µl of the sample were mixed with 100 µl of Protac® C at a concentration such that the content when activating Protein C was 0.17 U/ml, whereafter the sample was incubated at 37°C for 5 minutes for activation of PC.

200 µl of thrombin substrate S-2846 were then added together with a total of 200 µl of bovine FIX_a in Tris-buffer II, bovine FV_a in Tris-buffer I, Cephotest® and $CaCl_2$, the concentrations used being such as to obtain the final concentrations given below. The test solution obtained was incubated for 6 minutes at 37°C, whereafter the reaction was interrupted by adding 200 µl of 20%-acetic acid.

This process was carried out with plasma samples having the PC contents and final contents of the included components given below:

0% PC = Protein C-deficient plasma.
50% PC = A mixture of equal parts of normal plasma and Protein C-deficient plasma.

100% PC = Normal plasma.

Component	Content
Added human Protein S	0.11 µg/ml
FIX_a	16 pmol/l
FV_a	75 pmol/l
Cephotest®	4.2 percent by volume
Ca^{2+}	4.2 mmol/l

A_{405} was measured for these plasma and the values obtained are plotted against the PC contents (%) in Figure 5. It will be seen that good resolution was obtained, i.e. a high value of ΔA_{405} (0-100%) of 0.65.

The Figure also shows that the quantity of thrombin formed is clearly and inversely correlated to the proportion of Protein C in plasma. This is also supported by the fact that, as expected, no APC-activity whatsoever was obtained when plasma samples were preincubated with polyclonal anti-PC-antibodies for 5 minutes prior to the activating with Protac® C.

Example 8

The method has been tested clinically, where the method according to Example 1 (a) was carried out with plasma from an individual having normal Protein S content and with plasma from a thrombosis patient. For control purposes, the free Protein S content was also assayed by a conventional RIA-method ("Radio Immuno Assay").

The result achieved when practicing the invention was 115% PS and 53% PS respectively, which is in good agreement with the RIA values of 100% PS and 41% PS respectively.

CLAIMS

1. A method for determining the functional activity of free Protein S or Protein C in a plasma sample, c h a r a c t e r i z e d by adding to the plasma sample coagulation enzyme FIX_a, which enzyme promotes the formation of coagulation enzyme FX_a, optionally together with further coagulation reagent(s), incubating the sample, measuring in a known manner the quantity of thrombin formed from prothrombin, this process being promoted by FX_a; and determining the proportion of functionally active Protein S or Protein C, which is inversely correlated to the quantity of thrombin formed, on the basis of the measured quantity of thrombin, in a known manner with the aid of a standard.

2. A method according to Claim 1, c h a r a c t e r i z e d by adding to the sample as said further coagulation reagent(s) when determining Protein S, active Protein C (APC) or Protac® C prior to adding FIX_a, and when determining Protein C adding Protac® C and human Protein S to the plasma sample, and by preferably incubating the plasma sample subsequent to adding Protac® C but prior to adding FIX_a.

3. A method according to Claim 1 or 2, c h a r a c t e r i z e d by carrying out the method in one or in two stages, and by measuring the quantity of thrombin formed by means of a method based on substrate cleaving or a method based on coagulation.

4. A method according to Claim 1, 2 or 3, c h a r a c t e r i z e d by using a chromogenic thrombin substrate when applying the method based on substrate cleaving, and measuring the quantity of thrombin

obtained photometrically.

5. A method according to Claim 4, c h a r a c - t e r i z e d by using a chromogenic thrombin substrate which will enable the method to be carried out in one stage, said substrate preferably comprising H-D-Ala-Pro-Arg-pNa, H-D-CHG-Ala-Arg-pNA, tosyl-Gly-Pro-Arg-pNA, HD-CHT-Ala-Arg-pNA or H-D-CHG-But-Arg-pNA.

6. A method according to Claim 3, c h a r - a c t e r i z e d by using in the substrate cleaving method a plasma sample having a final plasma content of 0.02-10 percent by volume, and preferably 0.1-2 percent by volume.

7. A method according to any one of the preceding Claims, c h a r a c t e r i z e d in that FIX_a is of bovine origin.

8. A method according to any one of the preceding Claims, c h a r a c t e r i z e d by adding a phospholipid mixture and optionally one or more of the following coagulation factors: FV, FV_a, FVIII, $FVIII_a$, FX and prothrombin.

9. A method according to any one of the preceding Claims, c h a r a c t e r i z e d in that FIX_a and other coagulation proteins are of mammal origin, such as bovine, human or porcine origin, or may be formed by recombinant technique from native or genetically modified variants of these factors.

10. A method according to any one of the preceding Claims, c h a r a c t e r i z e d by preincubating the plasma sample with polyclonal or monoclonal

antibodies against FVIII, with the intention of inactivating FVIII.

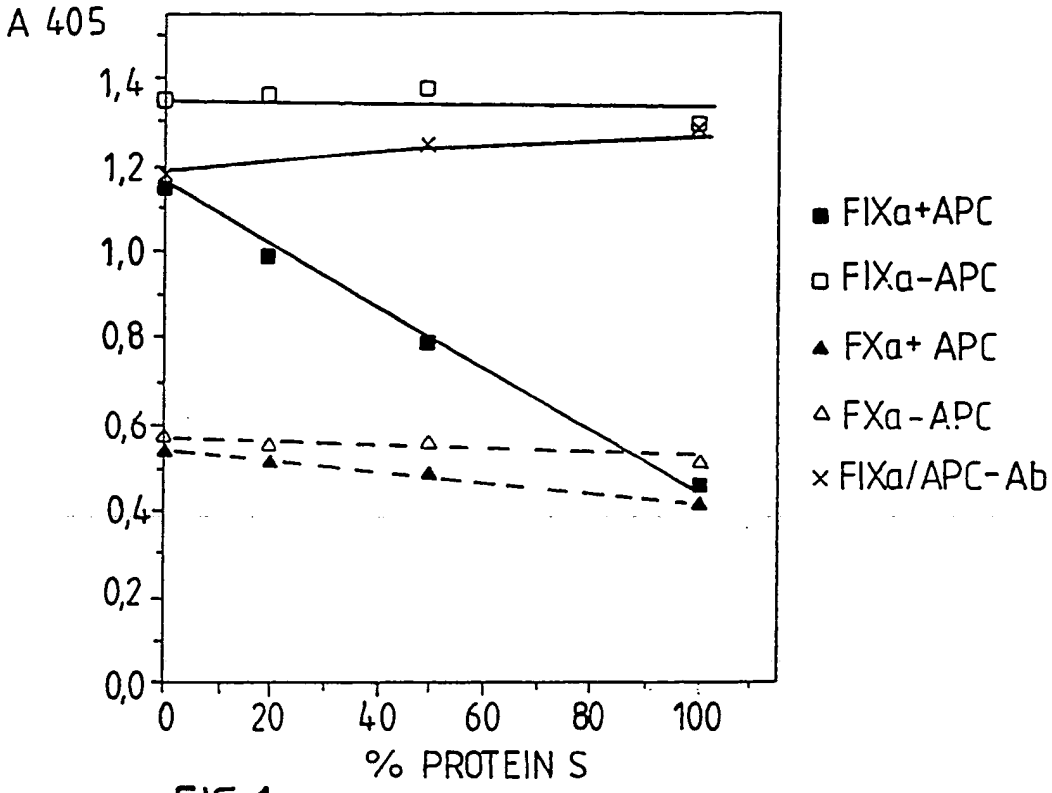

A 405
1,4
1,2
1,0
0,8
0,6
0,4
0,2
0,0
0
20
40
60
80
100
% PROTEIN S
■ FIXa+APC
□ FIXa-APC
▲ FXa+ APC
△ FXa-APC
× FIXa/APC-Ab

FIG.1

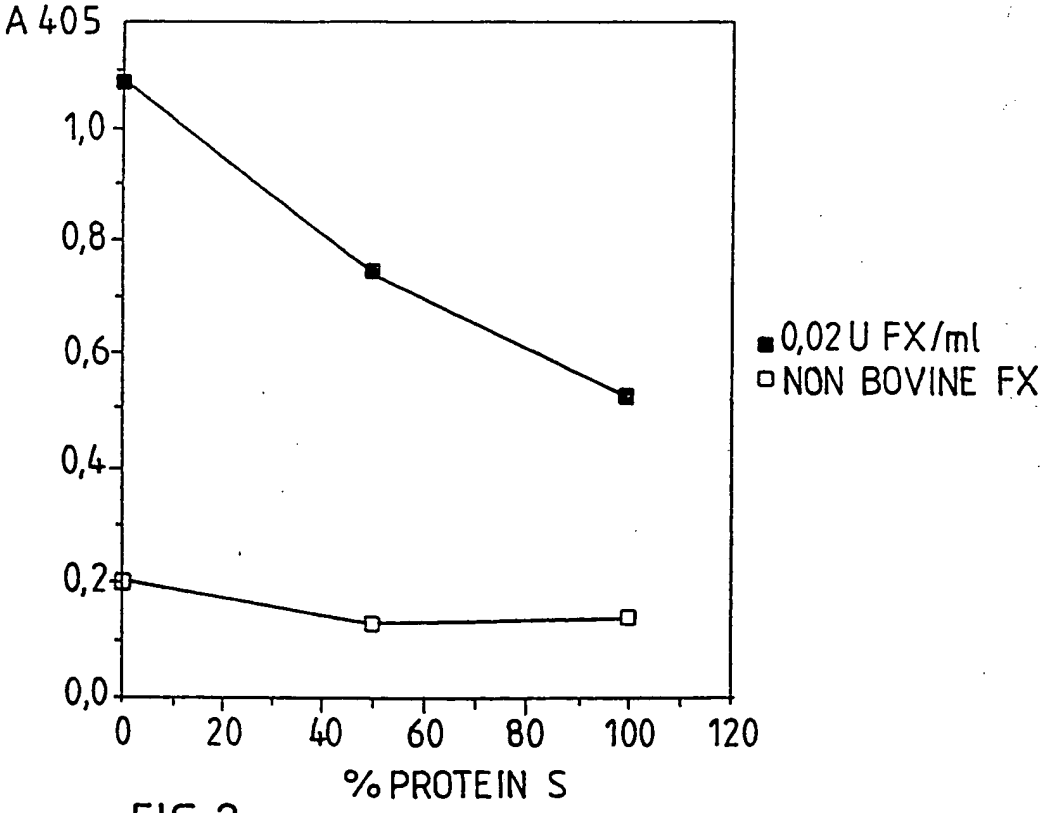

A 405
1,0
0,8
0,6
0,4
0,2
0,0
0
20
40
60
80
100
120
% PROTEIN S
■ 0,02 U FX/ml
□ NON BOVINE FX

FIG.2

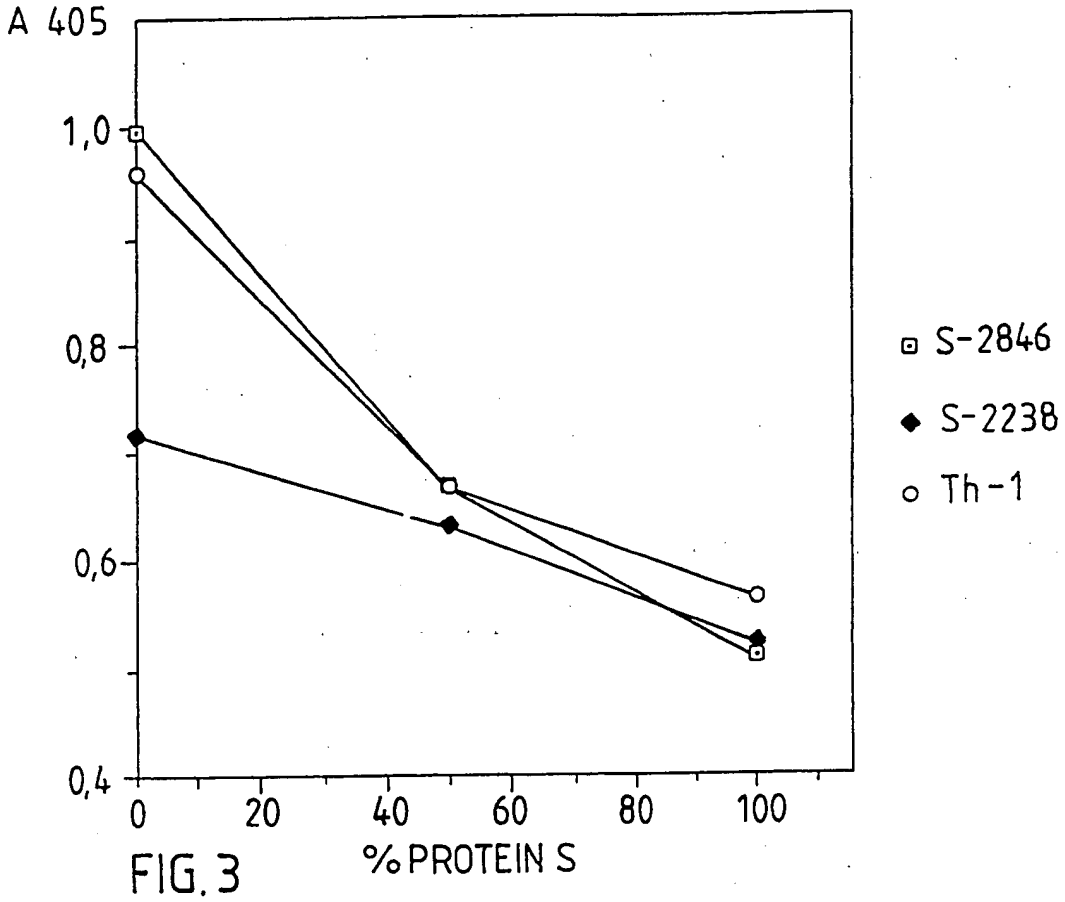

A 405
1,0
0,8
0,6
0,4
0
20
40
60
80
100
% PROTEIN S
S-2846
S-2238
Th-1

FIG. 3

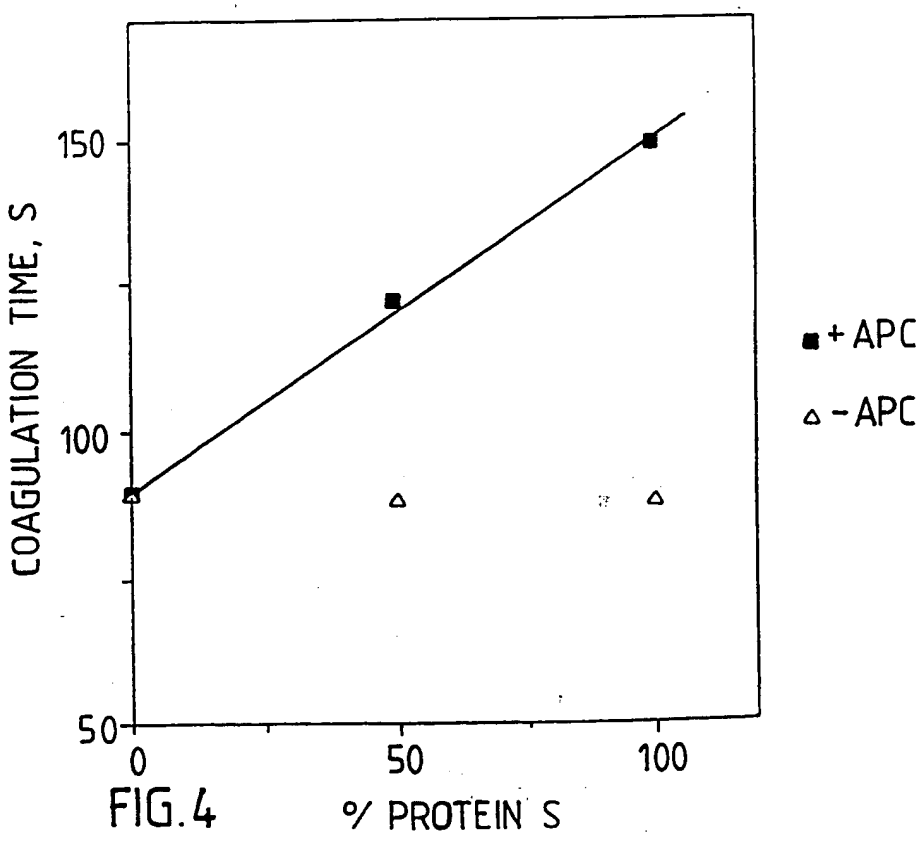

COAGULATION TIME, S
150
100
50
0
50
100
% PROTEIN S
+ APC
- APC

FIG. 4

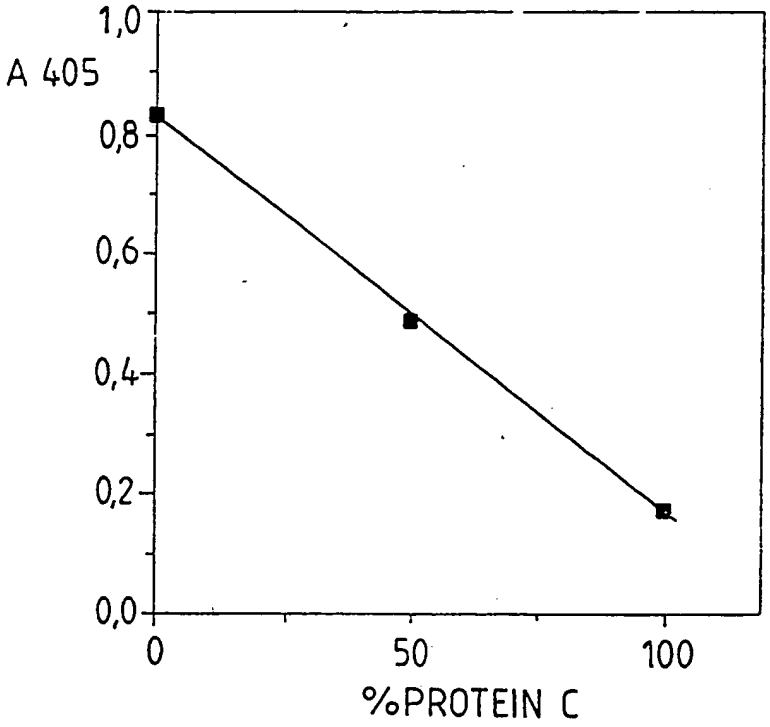


FIG.5

INTERNATIONAL SEARCH REPORT

International Application No PCT/SE 90/00478

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC5: C 12 Q 1/56, G 01 N 33/86

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
IPC5	C 12 Q; G 01 N

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in Fields Searched[8]

SE,DK,FI,NO classes as above

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category *	Citation of Document,[11] with indication, where appropriate, of the relevant passages[12]	Relevant to Claim No.[13]
A	Thrombosis Research, Vol. 41, 1986 Christine M. Hickton: "A functional assay of protein C in human plasma ", see page 501 - page 508 --	10
A	DE, A1, 3724443 (MAX-PLANCK-GESELLSCHAFT) 2 February 1989, see the whole document --	1-10
A	Blut, Vol. 52, 1986 Wilfried Thiel et al: "A Simplified Functional Assay for Protein C in Plasma Samples ", see page 169 - page 177 --	1-10
A	EP, A2, 0236985 (BOEHRINGER MANNHEIM GMBH) 16 September 1987, see the whole document --	1-10

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* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

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"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance, the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance, the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
11th October 1990	1990 -10- 12
International Searching Authority: SWEDISH PATENT OFFICE	Signature of Authorized Officer: Yvonne Siösteen

Form PCT/ISA/210 (second sheet) (January 1985)

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. PCT/SE 90/00478

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
The members are as contained in the Swedish Patent Office EDP file on 90-08-28
The Swedish Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
DE-A1- 3724443	89-02-02	NONE		
EP-A2- 0236985	87-09-16	AU-B-	577975	88-10-06
		AU-D-	6978587	87-09-10
		DE-A-	3607559	87-09-10
		JP-A-	62212569	87-09-18